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Glycoprotein D Homologs in Herpes Simplex Virus Type 1, Pseudorabies Virus, and Bovine Herpes Virus Type 1 Bind Directly to Human HveC (Nectin-1) with Different Affinities

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Distinct subsets of human receptors for alphaherpesviruses mediate the entry of herpes simplex virus (HSV), pseudorabies virus (PrV), or bovine herpes virus type 1 (BHV-1) into cells. Glycoprotein D (gD) is essential for receptor-mediated entry of all three viruses into cells. However, the gD homologs of these viruses share only 22–33% amino acid identity. Several entry receptors for HSV have been identified. Two of these, HveA (HVEM) and HveC (nectin-1), mediate entry of most HSV-1 and HSV-2 strains and are bound directly by HSV gD. A third receptor, HveB (nectin-2), mediates entry of HSV-2 and only a limited number of HSV-1 strains. HveB and HveC can also serve as entry receptors for PrV, whereas only HveC can serve this function for BHV-1. We show here that gD from PrV and BHV-1 binds directly to the human receptors that mediate PrV and BHV-1 entry. We expressed soluble forms of PrV gD and BHV-1 gD using recombinant baculoviruses and purified each protein. Using ELISA, we detected direct binding of PrV gD to HveB and HveC and direct binding of BHV-1 gD to HveC. Biosensor analysis revealed that PrV gD had a 10-fold higher affinity than HSV-1 gD for human HveC. In contrast, the binding of BHV-1 gD to HveC was weak. PrV gD and HSV-1 gD competed for binding to the V domain of HveC and both inhibited entry of the homologous and heterologous viruses. These data suggest that the two forms of gD bind to a common region on human HveC despite their low amino acid similarity. Based on affinities for human HveC, we predict a porcine HveC homolog may be important for PrV infection in its natural host, whereas a BHV-1 infection in its natural host may be mediated by a receptor other than a bovine HveC homolog.

Key Words: glycoprotein gD; HveC; nectin-1; HveB; nectin-2; HSV; BHV-1; PrV; entry.

INTRODUCTION

Herpes simplex virus (HSV), pseudorabies virus (PrV), and bovine herpes virus (BHV) are highly related alphaherpesviruses. In their natural hosts, HSV, PrV, and BHV typically cause lesions on mucosa and spread to the peripheral nervous system to establish latent infections in neurons. The viruses encode homologs of several membrane glycoproteins and at least four of these (gD, gH, gL, and gB) are essential for entry of virions into cells. The current model for virus entry involves a series of events. First gC and/or gB bind cell surface heparan sulfate proteoglycans. This is followed by the binding of gD to one of several cell surface receptors. The binding of gD to receptor is a critical step of viral entry that leads to membrane fusion facilitated by gD, gH, gL, and gB (see reviews in Enquist *et al.,* 1998; Spear, 1993; Spear *et al.,* 2000; Tikoo *et al.,* 1995).

Several cellular receptors for HSV have been identified through expression cloning and homology searches. Though initially identified as receptors for HSV, subsets of these receptors also mediate entry of PrV and BHV-1. Herpes virus entry mediator A (HveA or HVEM), a member of the tumor necrosis factor receptor family, can mediate entry of most HSV-1 and HSV-2 strains (Montgomery *et al.,* 1996). HveB, a member of the immunoglobulin (Ig) superfamily, can mediate entry of HSV-2, PrV, and some laboratory strains of HSV-1 (ANG, Rid) (Lopez *et al.,* 2000; Warner *et al.,* 1998). HveC, also a member of the Ig superfamily, can mediate entry of HSV-1, HSV-2, PrV, and BHV-1 (Cocchi *et al.,* 1998b; Geraghty *et al.,* 1998). HveB and HveC appear to be involved in cell–cell adhesion interactions and have been named nectin-2 and nectin-1, respectively (Aoki *et al.,* 1997; Lopez *et al.,* 1998; Takahashi *et al.,* 1999).

Expression of gD is limited to the alphaherpesvirus subfamily of herpesviruses and most alphaherpesviruses express gD. Though the function of gD in HSV-1, PrV, and BHV-1 entry appears similar, the three forms of gD share less than 33% amino acid identity. This sequence variability may contribute to differences in gD usage among the three viruses. Although gD is critical for entry of all three viruses, gD is required for direct cell-to-cell spread of HSV and BHV-1, but not PrV (Fehler

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et al., 1992; Ligas and Johnson, 1988; Rauh and Mettenleiter, 1991). Additionally, a gD-null BHV-1 that carries a gH mutation can overcome the need for gD to spread (Schröder *et al.,* 1997). In fact, mutants of both BHV-1 and PrV have been selected that overcome the need for gD entirely (Schmidt *et al.,* 1997; Schröder *et al.,* 1997).

Previous studies showed that expression of HSV, PrV, or BHV-1 gD inhibits the entry of both homologous and heterologous viruses, presumably because the expressed gD interacts with receptor(s) on the cell to inhibit productive interaction of the receptor with virus (Campadelli-Fiume *et al.,* 1988; Chase *et al.,* 1990, 1993; Johnson and Spear, 1989; Petrovskis *et al.,* 1988; Tikoo *et al.,* 1990). However, this gD-mediated interference is not always reciprocal. For example, bovine cells expressing BHV-1 gD were resistant to PrV infection, while cells expressing PrV gD were not resistant to BHV-1 (Chase *et al.,* 1990, 1993). This nonreciprocity may result from the use of different subsets of receptors by the viruses. Recently this issue was reexamined using Chinese hamster ovary (CHO) cells engineered to coexpress human HveC and one of the three gD molecules (Geraghty *et al.,* 2000). On these cells, HSV-1, PrV, and BHV-1 gD-mediated interference was reciprocal. All three gD molecules were shown to associate with HveC and inhibit entry of all three viruses. However, the extent of interference differed among the three forms of gD, which may reflect differences in affinity of the three forms of gD for HveC.

Soluble forms of HSV gD can also inhibit virus entry into cells, presumably by competing with virion gD for interaction with receptor(s) (Johnson *et al.,* 1990). Truncated soluble forms of HSV gD were previously shown to bind directly to receptors (Cocchi *et al.,* 1998a; Krummenacher *et al.,* 1998; Whitbeck *et al.,* 1997). The binding of gD from different HSV strains to either HveA or HveC directly correlates with the ability of those HSV strains to enter cells via the receptors (Geraghty *et al.,* 1998; Montgomery *et al.,* 1996). The goal of this study was to determine whether PrV gD and BHV-1 gD bind directly to receptors capable of mediating PrV and BHV-1 entry. Furthermore, we wanted to determine whether HSV-1 gD, PrV gD, and BHV-1 gD bind common receptors with different affinities. We found a correlation between the ability of HveA, HveB, and HveC to mediate entry of the viruses and the binding of the respective gD molecules to the receptors. We also found that PrV gD and HSV-1 gD bind to similar regions on HveC, but PrV gD binds with a higher affinity, causing it to compete better for HveC binding both in solution and on the cell surface.

RESULTS

Production and characterization of soluble gD proteins

We expressed and purified truncated soluble forms of PrV gD and BHV-1 gD using a baculovirus expression system (Fig. 1) (Sisk *et al.,* 1994; Willis *et al.,* 1998a). PrV gDt and BHV-1 gDt migrate as single bands by SDS–PAGE as judged by silver staining (Fig. 2A). Both PrV gDt and BHV-1 gDt were resistant to digestion by endoglycosidase H. BHV-1 gDt was endoglycosidase F sensitive, while PrV gDt was not (Fig. 2C). This indicates that BHV-1 gDt contains complex N-linked glycosylations, whereas PrV gDt contains no N-linked oligosaccharides, as predicted from the DNA sequences (Petrovskis *et al.,* 1986; van Drunen Littel-van den Hurk and Babiuk, 1985). Mass spectroscopy of the PrV gDt and BHV-1 gDt proteins reveals molecular masses of 40.7 and 41.5 kDa, respectively (Fig. 2B).

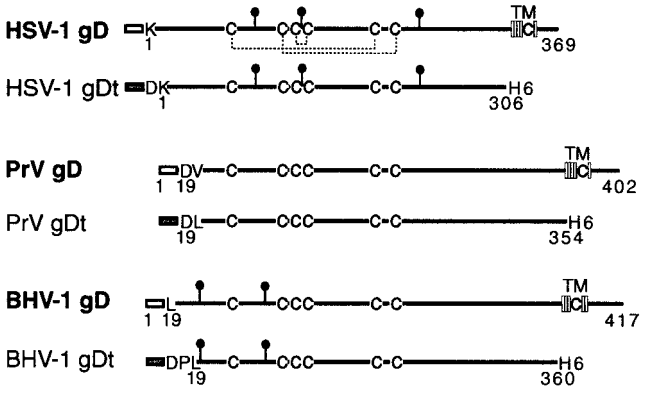

FIG. 1. Schematic representation of gD homologs. Full-length and truncated (t) forms of HSV-1 gD, PrV gD, and BHV-1 gD are depicted as solid lines. HSV-1 gD amino acids are numbered from the N-terminus of the mature gD after signal peptide cleavage. PrV gD and BHV-1 gD amino acids are numbered from the initial methionine. Open boxes represent the natural signal sequences. Gray boxes represent the mellitin signal peptide used in the baculovirus constructs. Cysteine residues are depicted as C's and disulfide bonds are indicated by dotted lines. Lollypop structures represent consensus N-glycosylation sites. Amino acid changes resulting from cloning are defined. Truncated proteins are tagged with a six-histidine tail (H6) after truncation at the indicated amino acid, prior to the transmembrane region (TM). Previous studies refer to this HSV-1 gDt as gD-1(306t).

Detection of PrV gDt and BHV-1 gDt with MAbs

Prior to testing receptor binding activity, we used MAbs to determine whether the soluble proteins were antigenically correct. Native or denatured PrV gDt or BHV-1 gDt were immobilized on nitrocellulose and probed with various MAbs (see Materials and Methods for MAb sources). PrV gDt reacted with two MAbs that recognized linear epitopes (6D8MB4, 87W001) and two MAbs that required native conformation for maximum reactivity (S4B1.1C11, 22M24). BHV-1 gDt reacted with four MAbs that recognized linear epitopes (9D6, 3D9S, 3402, 1B8-F11) and 15 MAbs that required native conformation for maximum reactivity (136, 3E7, 10C2, 4C1, 2C8, 3C1, 722, 1106, 2704, 4603, 5006, 1102, 1004, 4906, 2H6-C2) (data not shown).

In addition, we immunoprecipitated HSV-1, PrV, and

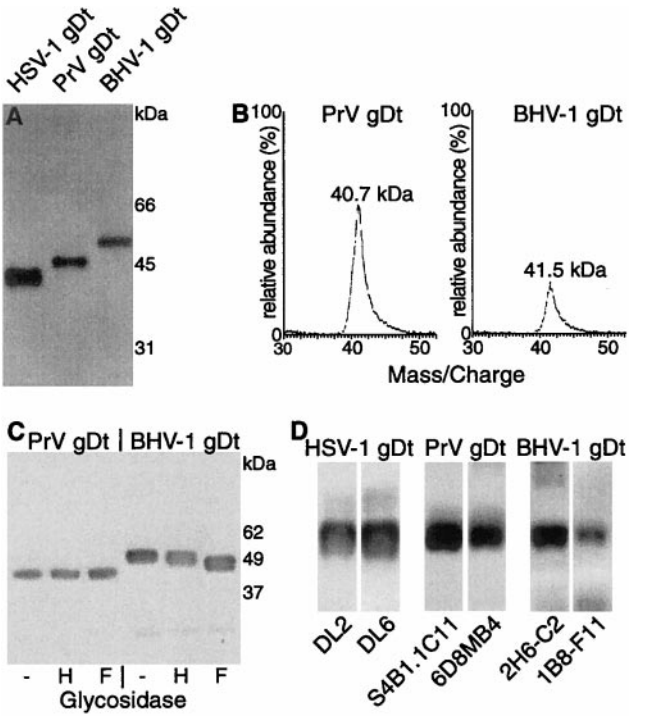

FIG. 2. Purified soluble PrV gDt and BHV-1 gDt. (A) HSV-1 gDt, PrV gDt, and BHV-1 gDt were purified from recombinant baculovirus-infected Sf9 cell supernatants, electrophoresed on denaturing SDS–10% polyacrylamide gels, and visualized by silver stain. Molecular mass markers are indicated. (B) Mass spectrometric analysis of PrV gDt and BHV-1 gDt was performed. Masses at peaks are indicated in kilodaltons (kDa). (C) PrV gDt and BHV-1 gDt were denatured; incubated overnight at 37°C with endoglycosidase H (lanes H), endoglycosidase F (lanes F), or no enzyme (lanes –); electrophoresed on denaturing SDS–10% polyacrylamide gels; and visualized by Western blot probed with anti-tetra-His MAb. Molecular mass markers are indicated. (D) HSV-1 gDt, PrV gDt, or BHV-1 gDt was immunoprecipitated using 8 μL of the indicated MAb, electrophoresed on denaturing SDS–10% polyacrylamide gels, and visualized by Western blots using PAb R7, R170, or EnvA. MAbs DL2, S4B1.1C11, and 2H6-C2 recognize conformational epitopes, while MAbs DL6, 6D8MB4, and 1B8-F11 recognize linear epitopes.

BHV-1 gDt using MAbs specific for conformational or linear epitopes (Fig. 2D). By adding increasing amounts of ascites fluid, we determined the maximum amount of gDt that could be immunoprecipitated using MAbs specific for linear epitopes (DL6, 6D8MB4, 1B8-F11). The amount of gDt immunoprecipitated by MAbs that recognize conformational epitopes (DL2, S4B1.1C11, 2H6-C2) was at least as great as the maximum amount immunoprecipitated by MAbs specific for linear epitopes. Thus, we conclude that the MAbs recognizing conformational epitopes immunoprecipitate at least as much gDt from our preparations as the MAbs specific for linear epitopes. Though the affinity of each MAb for gDt affects these results, the high efficiency of gDt immunoprecipitation using MAbs that recognize conformational epitopes increases our confidence that a large portion of the protein in our preparations is correctly folded.

Binding of PrV gDt and BHV-1 gDt to receptors by ELISA

We previously used ELISA to show that HSV-1 gDt binds directly and specifically to human HveA and HveC (Krummenacher *et al.,* 1998; Whitbeck *et al.,* 1997). Here we used ELISA to determine whether PrV gDt and BHV-1 gDt bind directly to HveA, HveB, or HveC. Purified HveAt, HveBt, or HveCt (Krummenacher *et al.,* 1998; Warner *et al.,* 1998; Whitbeck *et al.,* 1997) were immobilized on an ELISA plate and increasing concentrations of PrV gDt, BHV-1 gDt, or HSV-1 gDt were added. Bound gDt was then detected using antibodies. As expected, HSV-1 gDt bound to HveAt and HveCt in a dose-dependent fashion but did not bind HveBt (Fig. 3A). PrV gDt bound to HveCt and HveBt, but not to HveAt (Fig. 3B). BHV-1 gDt bound to HveCt, only weakly to HveBt, and not to HveAt (Fig. 3C). By comparing the binding of BHV-1 gDt and HSV-1 gCt to HveCt, we demonstrate that the binding of BHV-1 gDt to HveCt is specific (Fig. 3D). Thus, the ability of these

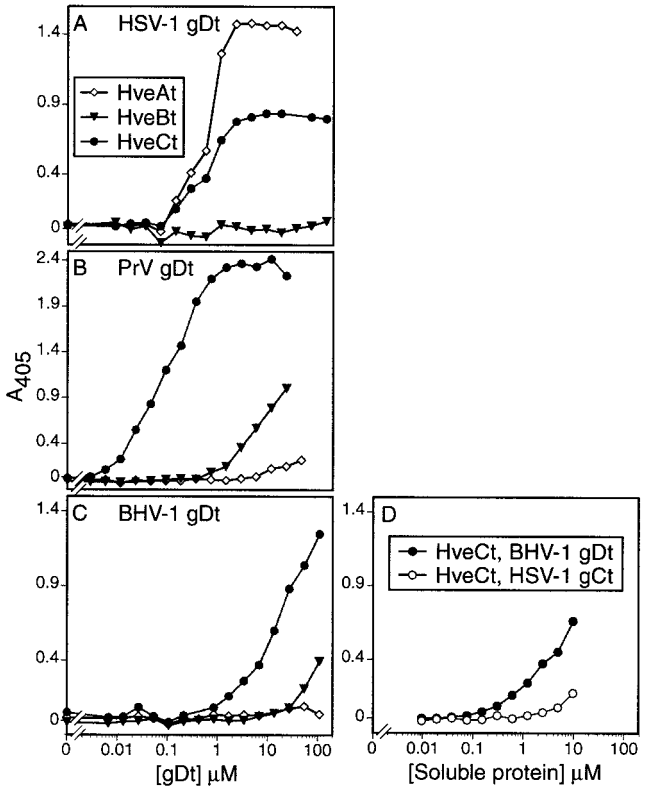

FIG. 3. Binding of gDt homologs to three HSV receptors. ELISA plates were coated with HveAt, HveBt, or HveCt. Increasing concentrations of HSV-1 gDt, PrV gDt, BHV-1 gDt, or HSV-1 gCt were added and binding was detected using specific antibodies. Absorbance at 405 nm (A_{405}) was recorded after the addition of HRP-conjugated secondary Ab followed by substrate. (A) HSV-1 gDt was detected using rabbit antiserum R7. (B) PrV gDt was detected using a mixture of MAbs S4B1.1C11 and 6D8MB4. (C) BHV-1 gDt was detected using a mixture of MAbs 3D9S, 4906, 3402, 10C2, and 9D6. (D) BHV-1 gDt and HSV-1 gCt binding to HveCt was detected using rabbit antisera PAb specific for tgD (BHV-1 gDt) or R47 (HSV-1 gCt).

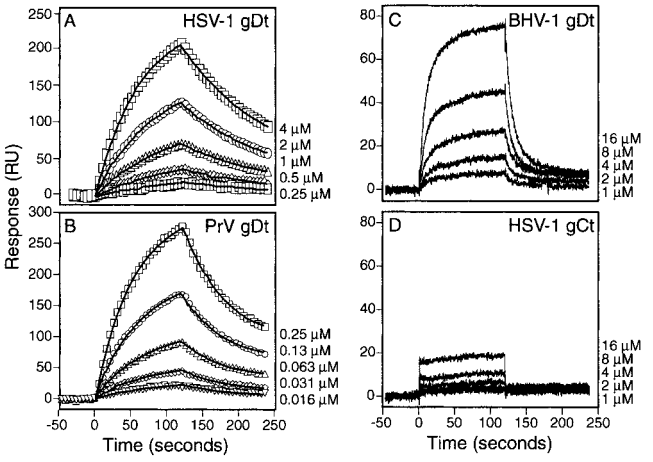

FIG. 4. Real-time analysis of gDt homologs binding HveCt. HveCt was immobilized on a CM5 chip in a BIAcore X instrument. The indicated concentrations of HSV-1 gDt, PrV gDt, BHV-1 gDt, and HSV-1 gCt were flowed over the chip for 2 min (association). Buffer alone was then flowed over the chip for 2 min (dissociation). The sensorgrams shown represent raw data with signals from a blank control flow cell and signals from injections of buffer alone subtracted out. Data points were collected at 5 Hz and the data were analyzed using BIAevaluation 3.0 software. (A) HSV-1 gDt binding HveCt (Krummenacher *et al.,* 1999). (B) PrV gDt binding HveCt. For clarity in (A) and (B), one of every 25 collected data points is represented by a symbol. The solid lines represent the best fit of the data using global fitting. (C) BHV-1 gDt binding HveCt. (D) HSV-1 gCt binding HveCt. In (C) and (D), the solid lines represent the collected data. No fit is depicted.

receptors to mediate viral entry correlates with the ability of the receptors to bind the corresponding viral gD.

By comparing the positions of curves and the gDt concentrations required to reach half-saturating levels of binding, we conclude that PrV gDt dinds HveCt with the highest affinity, followed by HSV-1 gDt and then BHV-1 gDt. Half-saturation of HveCt binding was reached at 0.08 μM PrV gDt and 0.6 μM HSV-1 gDt. Saturation of binding was not reached for BHV-1 gDt. PrV gDt also binds HveBt better than does BHV-1 gDt, although both gDt molecules bind HveBt less well than HveCt. To more accurately compare the binding affinities of the three gD proteins for HveC, we employed optical biosensor analysis.

HveC binding affinity constants

Biosensor analysis was previously used to measure the affinity of HSV-1 gDt for HveAt and HveCt (Krummenacher *et al.,* 1999; Whitbeck *et al.,* 1999; Willis *et al.,* 1998b). Here we examined the binding affinities of PrV gDt and BHV-1 gDt for HveCt. Serial dilutions of HSV-1 gDt, PrV gDt, BHV-1 gDt, and HSV-1 gCt were flowed over HveCt immobilized on a chip and real-time binding was recorded (Fig. 4). HSV-1 gDt binding data were previously fit to a 1:1 binding model (Krummenacher *et al.,* 1999). PrV gDt binding data were fit to the same model.

Kinetic rates and affinity constants for the HSV-1 gDt and PrV gDt are summarized in Table 1. Data for HSV-1 gDt binding to HveCt were previously reported (Whitbeck *et al.,* 1999) and are included for comparison. PrV gDt had a 10-fold higher affinity for HveC than did HSV-1 gDt and this higher affinity was mainly the result of an increased on rate (k_{on}) of binding.

High concentrations of BHV-1 gDt were required to observe binding to HveCt on the chip (Fig. 4). For example, a binding signal of 70 resonance units (RU) required 16 μM BHV-1 gDt, but only 0.063 μM PrV gDt. Comparing equal concentrations of BHV-1 gDt and HSV-1 gCt binding to HveCt demonstrates that the binding of BHV-1 gDt to HveCt is specific. Smooth association and dissociation curves are seen with BHV-1 gDt and not with HSV-1 gCt. The HSV-1 gCt signals represent background changes in refractive index as a result of the high concentrations of protein used.

We were unable to calculate an affinity constant for BHV-1 gDt binding to HveCt because the data did not fit any model provided in the BIAevaluation software package. However, the shape of the binding curves suggests that BHV-1 gDt binds HveCt, but rapidly dissociates (fast off-rate). Thus, the weak binding is apparently the result of poor stability of the complex. We calculated the maximum off-rate for BHV-1 gDt at 34×10^{-2} s^{-1} (Rux *et al.,* 1998). We conclude from the biosensor and ELISA data that BHV-1 gDt has a significantly lower affinity for HveCt than does either HSV-1 gDt or PrV gDt. For this reason, we limited the following studies to the interaction between PrV gD and HveC.

Localization of PrV gDt binding site to HveC V domain

We previously showed that HSV-1 gDt binds directly to a soluble form of HveC containing only the immunoglobulin V domain, called HveC(143t) (Krummenacher *et al.,* 1999). To determine whether PrV gDt also binds to the V domain, HveC(143t) was immobilized on an ELISA plate and increasing concentrations of PrV gDt were added. Binding to HveC(143t) was observed, although high concentrations of PrV gDt were required (Fig. 5A).

Previous studies showed that recognition of

TABLE 1

Kinetic and Affinity Values for gDt/HveCt Complex Formation

	k_{on} (10^3 M^{-1} s^{-1}) (on rate)	k_{off} (10^{-2} s^{-1}) (off rate)	K_D (10^{-6} M)[a] (affinity)
HSV-1 gDt[b]	2.6 ± 0.7[c]	0.71 ± 0.09[c]	2.7 ± 0.2
PrV gDt	98 ± 5[c]	1.3 ± 0.1[c]	0.13 ± 0.07

[a] $K_D = k_{off}/k_{on}$.

[b] Values for HSV-1 gDt were reported previously (Whitbeck *et al.,* 1999) and are included here for comparison.

[c] Average ± SD from at least three experiments.

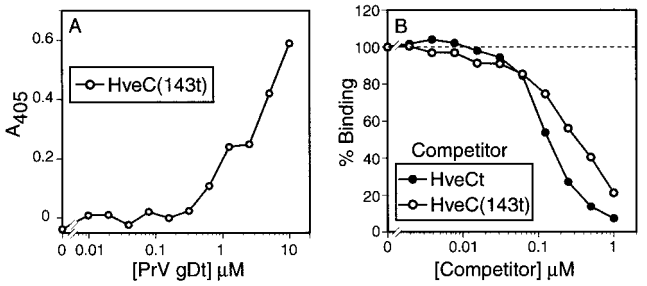

FIG. 5. PrV gDt binding to the V domain of HveC. (A) ELISA plates were coated with HveC(143t) and incubated with increasing concentrations of PrV gDt. Bound PrV gDt was detected using a mixture of MAbs S4B1.1C11 and 6D8MB4. (B) ELISA plates were coated with HveCt and incubated with a constant concentration of PrV gDt and increasing concentrations of either HveCt or HveC(143t) (as soluble competitors). PrV gDt bound to immobilized HveCt was detected with a mixture of MAbs S4B1.1C11 and 6D8MB4. Results are expressed as a percentage of gD binding in the absence of soluble competitor (100%).

HveC(143t) by a conformational MAb is reduced when HveC(143t) is immobilized on an ELISA plate (Krummenacher *et al.,* 1999). To compare the binding of PrV gDt to HveCt and HveC(143t), we used a competition assay that measures the binding of PrV gDt and HveCt in solution. ELISA plates coated with HveCt were incubated with a mixture of PrV gDt and increasing concentrations of either HveC(143t) or HveCt. PrV gDt binding to the immobilized HveCt was then measured. The amount of binding in the absence of soluble competitor was defined as 100% binding. Soluble HveCt and HveC(143t) inhibited the binding of PrV gDt to the immobilized HveCt equally well (Fig. 5B), indicating that PrV gDt binds to the V domain of HveC as efficiently as it does to the full ectodomain of HveC.

Competition between HSV-1 and PrV gDt for HveC binding

Considering that both HSV-1 gDt and PrV gDt bind to the HveC V domain (Cocchi *et al.,* 1998a; Krummenacher *et al.,* 1999), we determined whether the two gD proteins could compete for HveCt binding. HveCt was immobilized on an ELISA plate and mixtures of the two gD proteins were added in solution. We determined whether increasing the concentration of one gDt affected the binding of the second gDt held at a constant concentration. The amount of binding in the absence of soluble competitor was defined as 100% binding. As the concentration of PrV gDt increased, the binding of HSV-1 gDt decreased (Fig. 6A). Likewise, as the concentration of HSV-1 gDt increased, the binding of PrV gDt decreased (Fig. 6B). We conclude that PrV gDt and HSV-1 gDt compete with each other for HveCt binding.

HSV-1 gDt binding was inhibited by 50% at 0.55 μM PrV gDt and PrV gDt binding was inhibited by 50% at 3.5 μM HSV-1 gDt. This observation agrees with the previous data indicating that PrV gDt has a higher affinity for HveCt than does HSV-1 gDt.

Inhibiting infection of HveC-expressing cells with soluble gDt

Previous reports showed that soluble HSV gD can inhibit HSV entry, presumably by competing with virion gD for interaction with receptor (Fuller and Lee, 1992; Johnson *et al.,* 1990; Nicola *et al.,* 1997; Tal-Singer *et al.,* 1995). We used this type of inhibition of entry to examine how HSV-1 gDt and PrV gDt compete with virion gD to bind to HveC on a cell surface. Since HSV-1 gDt and PrV gDt compete to bind to HveCt, both forms of gDt should inhibit entry of both HSV-1 and PrV into HveC-expressing cells. Furthermore, if the two gD molecules differ in affinity for full-length HveC on the cell surface, PrV gDt should inhibit virus entry more efficiently than does HSV-1 gDt. To test this, M3A cells, which are derived from nonpermissive CHO cells and express human HveC, were incubated with HSV-1 gDt or PrV gDt prior to the addition of HSV-1 hrR3 or PrV BeBlu, virus strains carrying *lacZ* (Banfield *et al.,* 1998; Goldstein and Weller, 1988). The amount of entry in the absence of soluble protein was defined as 100% entry.

Entry of HSV-1 and PrV into M3A cells was efficiently inhibited by both gDt proteins (Fig. 7). The inhibition of both homologous and heterologous virus entry by HSV-1 gDt and PrV gDt agrees with the competition ELISA results. Moreover, PrV gDt was more efficient than HSV-1

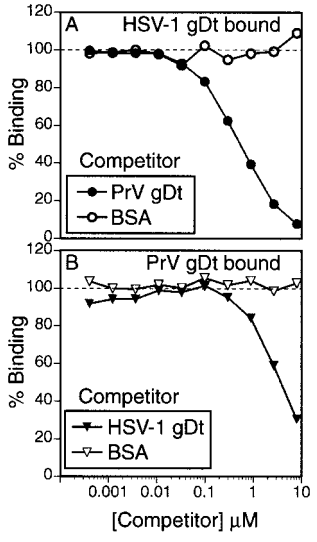

FIG. 6. Competition between PrV gDt and HSV-1 gDt for HveC binding. ELISA plates were coated with HveCt and incubated with mixtures of HSV-1 gDt and PrV gDt. Results are expressed as a percentage of binding in the absence of soluble competitor (100%). (A) HSV-1 gDt (0.5 μM) and increasing concentrations of PrV gDt (as competitor) were added to the plate. Bound HSV-1 gDt was detected with MAb DL2. (B) PrV gDt (0.5 μM) and increasing concentrations of HSV-1 gDt (as competitor) were added to the plate. Bound PrV gDt was detected with MAb 6D8MB4.

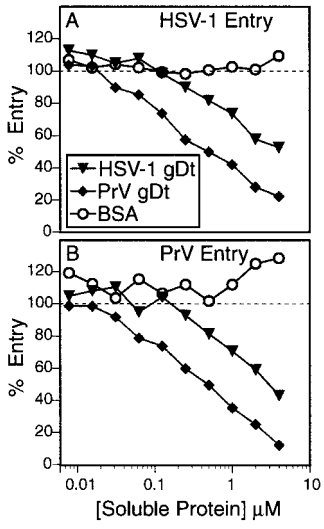

FIG. 7. Inhibition of HSV-1 and PrV entry with HSV-1 gDt and PrV gDt. M3A cells expressing HveC were incubated with gDt or BSA at 4°C for 90 min. Virus encoding *lacZ* was added and after 90 min, the cells were shifted to 37°C. After 5 h, the cells were lysed and β-gal activity was measured as an indication of virus entry. Signals from duplicate wells were averaged for each concentration and expressed as a percentage of signal in the absence of soluble protein (100%). (A) Inhibition of HSV-1 entry. (B) Inhibition of PrV entry.

gDt at inhibiting entry of both viruses. The concentrations required to inhibit 40% of both HSV-1 and PrV entry were 2 μM HSV-1 gDt and 0.25 μM PrV gDt. These results correlate with affinity differences measured using soluble HveCt. We conclude that the higher affinity of PrV gDt for HveC is evident both when HveC is on the cell surface and in a truncated soluble form.

Inhibiting PrV entry into porcine cells with PrV gDt

Since the preceding virus entry studies examined the interaction between PrV gD and a human receptor, we also tested the ability of PrV gDt to inhibit entry into porcine cells (Fig. 8). Porcine kidney (PK15) cells were incubated with increasing concentrations of PrV gDt and then infected with PrV BeBlu. PrV gDt effectively inhibited the entry of PrV. We conclude that soluble PrV gDt is able to inhibit PrV entry, presumably by interacting with a porcine entry receptor(s) and competing with viral gD for binding.

DISCUSSION

Previous studies showed that human HveC can mediate cell entry of HSV-1, PrV, and BHV-1, despite low amino acid similarity among the gD homologs of these viruses (Geraghty *et al.,* 1998). This study demonstrates direct binding of soluble forms of the three gD molecules to HveC. Furthermore, we detected differences in affinity for HveC among the three gD molecules.

Using a baculovirus expression system, we produced purified soluble forms of three gD homologs that retained receptor-binding capacity. Previously other gD homologs were also successfully expressed using baculovirus, including that of HSV, BHV-1, and equine herpesvirus (Flowers *et al.,* 1995; Sisk *et al.,* 1994; van Drunen Littel-van den Hurk *et al.,* 1991).

Direct binding of gD to receptors

Our ELISA binding data correlate with the known cell receptor usage for HSV-1, PrV, and BHV-1 (Geraghty *et al.,* 1998; Montgomery *et al.,* 1996; Warner *et al.,* 1998). Specifically, HveA mediates entry of only HSV and binds only HSV gDt. In contrast, HveC mediates entry of all three viruses and binds all three forms of gDt.

HveB was reported to mediate entry of only PrV, but it binds both PrV gDt and BHV-1 gDt. However, BHV-1 gDt binding is weak and requires high concentrations. This low level of binding may be insufficient to mediate entry. Both PrV gDt and BHV-1 gDt bind HveBt less well than HveCt. This lower binding capacity of HveB was also shown with an HSV-1 gD mutant protein that is able to bind both HveB and HveC (Lopez *et al.,* 2000).

The ELISA data suggest the affinity for HveCt is highest with PrV gDt, followed by HSV-1 gDt, and then with BHV-1 gDt. Biosensor data show that the high affinity of PrV gDt for HveCt is primarily because of a faster on-rate. The affinity of PrV gDt for HveC is 10-fold higher than the affinity of HSV-1 gDt for HveC. The off-rates for PrV gDt and HSV-1 gDt are similar, indicating that the stability of the gD/HveC complex is similar for both PrV gDt and HSV-1 gDt. The affinity of PrV gDt for HveC is comparable to that of gD(rid1t), a previously characterized HSV-1 gD mutant protein which carries a mutation at position 27 (Dean *et al.,* 1994; Krummenacher *et al.,* 1999).

We were unable to calculate a K_D for BHV-1 gDt binding HveCt; however, we estimate the affinity is at least 10-fold lower than that of HSV-1 gD (2.7×10^{-6} M) based on both ELISA and biosensor results. Biosensor data

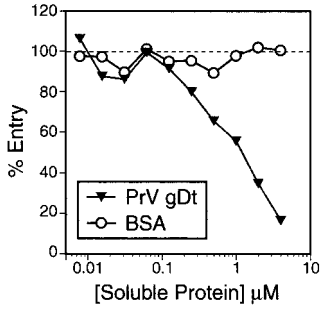

FIG. 8. Inhibition of PrV entry with PrV gDt. PK15 cells were incubated with PrV gDt or BSA at 4°C for 90 min. PrV encoding *lacZ* was added and after 90 min, the cells were shifted to 37°C. After 5 h, the cells were lysed and β-gal activity was measured as an indication of PrV entry. Signals from duplicate wells were averaged for each concentration and expressed as a percentage of signal in the absence of soluble protein (100%).

show a fast off-rate, suggesting that the BHV-1 gD/HveC complex is unstable. Similar biosensor data were obtained previously with an HSV-1 gD C-terminal truncation mutant called HSV gD(234t), which binds HveAt but dissociates rapidly (Rux *et al.,* 1998).

Assuming our *in vitro* results reflect the situation *in vivo,* these differences in affinity for HveC may explain why expression of different forms of gD confers different degrees of resistance to infection. The low affinity of BHV-1 gDt for HveC likely explains why expression of BHV-1 gD in HveC-expressing CHO cells inhibits viral entry less efficiently than expression of either HSV-1 gD or PrV gD (Geraghty *et al.,* 2000). Although expression of PrV gD in that study did not produce much higher levels of inhibition than those of HSV-1 gD, a previous study showed HeLa cells expressing PrV gD are more resistant to HSV than to PrV (Petrovskis *et al.,* 1988). Since HeLa cells express HveC (Cocchi *et al.,* 1998b), the high affinity of PrV gD for HveC may explain this greater resistance to infection by HSV than by Prv.

PrV gD and HSV-1 gD competition for HveC binding

PrV gDt binds to the V domain of HveC as efficiently as it does to the full ectodomain of HveC. This shows that PrV gDt and HSV-1 gDt bind to the same domain on HveC. Moreover, PrV gDt and HSV-1 gDt compete with each other to bind HveC by ELISA, suggesting that the two gD molecules bind the same physical structure on the V domain of HveC. Thus, PrV gD may bind to HveC within a critical HSV-1 gD binding site recently identified between amino acid residues 80 and 104 (Krummenacher *et al.,* 2000).

The binding of both forms of gD to the same region of HveC implies a common structure between these two homologs, despite their low amino acid similarity. The conservation of cysteine spacing may be a key element of this structure (Fig. 1).

The competition between HSV-1 gD and PrV gD for HveC binding is also seen when HveC is on the cell surface and gD is in the virion. HSV-1 gDt and PrV gDt inhibit entry of both HSV-1 and PrV on HveC-expressing CHO cells, presumably by competing with virion gD for binding to HveC on the cell. As in the competition ELISA, the affinity difference between PrV gDt and HSV-1 gDt is evident, with PrV gDt inhibiting entry of both HSV-1 and PrV more efficiently than does HSV-1 gDt.

Competition for HveC binding explains previous results showing that expression of HSV-1 gD or PrV gD interferes with infection by the heterologous virus (Geraghty *et al.,* 2000). Competition for HveC binding may also explain why UV-inactivated PrV added to cells is able to inhibit HSV-1 entry and vice versa (Lee and Fuller, 1993).

HveC homologs

PrV gDt inhibited entry of PrV when added to porcine cells prior to infection, presumably by competing with virion gD for binding to porcine cell surface receptor(s). This type of inhibition was previously shown with soluble forms of HSV gD and BHV-1 gD (Johnson *et al.,* 1990; Li *et al.,* 1995).

The porcine receptor(s) involved in PrV entry is unknown, but a murine HveC homolog with a remarkable level of conservation with human HveC was recently identified (Menotti *et al.,* 2000). Perhaps highly conserved bovine and porcine HveC homologs also exist. If PrV uses a porcine HveC homolog for entry, PrV gD binding likely involves the V domain of porcine HveC.

The low affinity and rapid dissociation of BHV-1 gDt from human HveC shed doubt on the importance of a bovine HveC homolog for BHV-1 infection. However, the affinity of BHV-1 gD for bovine HveC may be much higher than that for human HveC or virion gD avidity for receptor may affect HveC usage. Previous studies showed that PrV gD expression in bovine cells did not interfere with BHV-1 entry, implying that PrV and BHV-1 can use different receptors for entry (Chase *et al.,* 1993). The ability of BHV-1 gD expression to interfere with PrV entry implies the viruses can also share a receptor (Chase *et al.,* 1990). These results indicate BHV-1 likely uses multiple receptors for entry into bovine cells.

This study showed that human HveC is functional for alphaherpesvirus entry into cells, despite a wide range in affinities of gD for the receptor. Even low-affinity binding, such as that between BHV gDt and HveCt or PrV gDt and HveBt, is sufficient to mediate virus entry. The affinities of PrV gD and BHV-1 gD for their native receptors and the ability of porcine and bovine HveC homologs, if they exist, to serve as receptors remain to be determined.

MATERIALS AND METHODS

Cells and viruses

Sf9 (*Spodoptera frugiperda*) cells (GIBCO BRL, Gaithersburg, MD) were maintained in suspension in Sf900II medium (GIBCO BRL) or as a monolayer in Grace's medium (GIBCO BRL) supplemented with 10% fetal calf serum (FCS). CHO-IEβ8 cells express the β-galactosidase gene under the control of the ICP4 promoter and were kindly provided by Dr. P. G. Spear. M3A cells were derived from CHO-IEβ8 cells stably transfected with the gene for HveC under the cytomegalovirus IE promoter (Krummenacher *et al.,* 1999; Montgomery *et al.,* 1996; Terry-Allison *et al.,* 1998). CHO-IEβ8 cells were grown in Ham's F-12 medium supplemented with 10% FCS and 150 μg/mL puromycin. M3A cells were and grown in Ham's F-12 medium supplemented with 10% FCS, 150 μg/mL puromycin, and 250 μg/mL G418. PK15 cells (American

Type Culture Collection [ATCC], Rockville, MD) were grown in DMEM (Dulbecco's minimal essential medium) supplemented with 5% FCS.

HSV-1 hrR3 carries *lacZ* under the ICP6 promoter and was kindly provided by Dr. S. K. Weller (Goldstein and Weller, 1988). PrV BeBlu carries *lacZ* under the gG promoter and was kindly provided by Dr. L. W. Enquist (Banfield *et al.,* 1998).

Polyclonal (PAb) and monoclonal (MAb) antibodies

Anti-HSV-1 gD MAb DL2 was raised against full-length purified HSV-1 gD (Cohen *et al.,* 1986). Rabbit PAb serum R7 was raised against HSV-2 gD and cross-reacts with HSV-1 gD (Isola *et al.,* 1989). PrV gD MAbs were kindly provided by Drs. N. E. Coe (S4B1.1C11) (Coe and Mengeling, 1990), M. Eloit (22M24) (Eloit *et al.,* 1988), and F. A. Zuckermann (87W001) (Zuckermann *et al.,* 1988). Rabbit PAb serum R170 was raised against purified PrV gDt. BHV-1 gD MAbs were kindly provided by Drs. L. A. Babiuk and S. van Drunen Littel-van den Hurk (136, 9D6, 3E7, 10C2, 4C1, 2C8, 3C1, 3D9S) (Hughes *et al.,* 1988; van Drunen Littel-van den Hurk *et al.,* 1984) and by Dr. G. J. Letchworth (722, 2704, 4603, 5006, 1106, 4906, 1102, 1004, 3402) (Marshall *et al.,* 1988). Rabbit PAb serum (PAb specific for tgD) was raised against truncated BHV-1 gD and was kindly provided by Dr. S. van Drunen Littel-van den Hurk (Kowalski *et al.,* 1993). EnvA is a rabbit polyclonal serum raised against the Nonidet P-40 (NP-40)-soluble components of gradient-purified BHV-1 virions and was a generous gift from L. J. Bello and W. C. Lawrence. Hybridoma cell lines for the PrV gD MAb 6D8MB4 and BHV-1 gD MAbs 1B8-F11 and 2H6-C2 were obtained from ATCC. Ascites fluids were prepared by Cocalico Biologicals (Backwater, PA). Anti-tetra-His MAb IgG was obtained from Qiagen (Chatsworth, CA). Anti-HveAt MAb CW2 was raised against purified HveAt (Whitbeck *et al.,* 2000).

Construction of recombinant baculoviruses

The strategy used to generate PrV gDt and BHV-1 gDt was the same as described previously to generate soluble HSV gD-1(306t) (Sisk *et al.,* 1994). As a result of the cloning strategy, the predicted native signal sequences for PrV gD (residues 1–17) and BHV-1 gD (residues 1–18) were replaced with the honeybee mellitin signal sequence, the predicted transmembrane and cytoplasmic regions were removed, and the C-termini were tagged with six histidine residues. Plasmid pWW117 (Muggeridge *et al.,* 1990) was used as a template for PCR amplification of PrV gDt (Rice strain). An upstream primer 5′-CCGAGATCTGGACGCTGTGCCCGCACCGACCT-3′ and a downstream primer 5′-GCCGAATTCAATGATGATGGTGATGATGTGAGCGATGGCGCGAGA-3′ amplified a truncated version of PrV gD, including amino acids 19 to 354, with a valine-to-leucine change at residue 20. Plasmid BHV1*Hin*dIIIK (Lawrence *et al.,* 1986) was kindly provided by Dr. W.C. Lawrence and used as a template for PCR amplification of BHV-1 gDt (Cooper strain). An upstream primer 5′-GCGGATCCATTGCCTACACCTG-CACCACGGGTGAC-3′ and a downstream primer 5′-CGGAATTCAATGATGATGATGATGATGGGCGTCAGG-GGCTGCGGGCGTA-3′ amplified a truncated version of BHV-1 gD, including amino acids 19 to 360, with an aspartic acid and proline added at the N-terminus. Using the underlined restriction digest sites, the PrV gD and BHV-1 gD fragments were digested and ligated with DNA from pVT-Bac (Tessier *et al.,* 1991) to generate plasmids pSC322 and pSC323, respectively. DNA from these plasmids was recombined into baculovirus by cotransfection with Baculogold DNA (PharMingen, San Diego, CA) into Sf9 cells. Plaques were picked and screened for protein expression via Western blot. Baculovirus recombinants expressing gD were subjected to two rounds of plaque purification. The recombinant baculoviruses were named bac-PrVgDt and bac-BHV1gDt; the recombinant proteins were named PrV gDt and BHV-1 gDt.

Production and purification of PrV gDt and BHV-1 gDt

PrV gDt and BHV-1 gDt were produced and purified essentially as described for HSV gD-1(306t) (Sisk *et al.,* 1994; Willis *et al.,* 1998a). Sf9 cells were grown in suspension, infected with bac-PrVgDt or bac-BHV1gDt at a multiplicity of infection (m.o.i.) of 4, and the cells were removed by centrifugation at 48–72 h postinfection. The supernatant was filtered and the buffer was exchanged with phosphate-buffered saline (PBS, 20 mM sodium phosphate buffer containing 0.15 M NaCl) either by dialysis (12 to 14 kDa molecular mass cutoff) or by tangential flow filtration (10 kDa molecular mass cutoff membrane; Millipore, Bedford, MA). The supernatant was then incubated with nickel-nitriloacetic acid resin (Qiagen) overnight on a rotary shaker at 4°C. The resin was pelleted, resuspended in PBS, and transferred to a column. The proteins were eluted using increasing concentrations of imidazole buffer (0.01 to 0.25 M) in 20 mM sodium phosphate buffer (pH 7.5) containing 0.5 M NaCl. The bulk of purified PrV gDt and BHV-1 gDt eluted in 100–250 mM imidazole. These elution fractions were dialyzed (PBS) and concentrated using a 10 kDa molecular mass centrifugal membrane (Millipore). Total protein concentrations were determined by a Coomassie assay (Pierce, Rockford, IL). The yield of purified protein was 0.5 to 5 mg/L of supernatant.

Production and purification of HSV-1 gDt, HSV-1 gCt, and soluble receptors

The procedures for production and purification of HSV-1 gDt, HSV-1 gCt, HveAt, HveBt, HveCt, and HveC(143t) were described previously (Krummenacher *et al.,* 1998, 1999; Sisk *et al.,* 1994; Tal-Singer *et al.,* 1995;

Warner *et al.,* 1998; Whitbeck *et al.,* 1997). For clarity, the truncated forms HSV gD-1(306t), HSV gC-1(457t), HveA(200t), HveB(361t), and HveC(346t) are referred to here as HSV-1 gDt, HSV-1 gCt, HveAt, HveBt, and HveCt, respectively. These proteins were truncated just prior to the transmembrane region and tagged with six-His tails. HveC(143t) was truncated C-terminal to the V domain of HveC and tagged with a six-His tail. The molecular weights of HSV-1 gDt and HSV-1 gCt are 37.2 and 58 kDa, respectively.

SDS–PAGE analysis

Purified glycoproteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions in precast Tris–glycine gels (Novex, San Diego, CA). Following SDS–PAGE, the proteins were either stained with silver nitrate (Pharmacia Laboratories, Piscataway, NJ) or transferred to nitrocellulose and incubated in PBS containing 5% nonfat dry milk and 0.2% Tween 20 (blocking solution). Blots were reacted with various MAbs and incubated with secondary antibody (goat anti-mouse or goat anti-rabbit) coupled to horseradish peroxidase (HRP). Blots were washed and visualized by exposure to film after the addition of chemilumenescent substrate (ECL; Amersham, Arlington Heights, IL).

Antigenic analysis by dot blot

PrV gDt, BHV-1 gDt, and BSA were denatured by boiling in SDS and β-mercaptoethanol. Equivalent amounts of denatured and native protein were immobilized onto nitrocellulose using a dot-blot apparatus. Blots were incubated in blocking solution and reacted overnight at 4°C with MAbs at a dilution of 1:500. Blots were then visualized as described above. MAbs were classified as requiring native conformation for maximal reactivity if they reacted with native protein better than with denatured protein.

Immunoprecipitations

Samples (300 ng) of HSV-1 gDt, PrV gDt, or BHV-1 gDt were incubated on ice with 1, 2, 4, or 8 μL of MAb ascites fluid in 50 μL binding buffer (10 mM Tris, pH 8.0; 100 mM NaCl; 0.1% NP-40; 0.05% BSA; 0.05% chicken egg albumin). HSV-1 gDt was incubated with MAb DL2 or DL6. PrV gDt was incubated with MAb 6D8MB4 or S4B1.1C11. BHV-1 gDt was incubated with MAb 1B8-F11 or 2H6-C2. As a negative control, each gDt was also incubated with MAb CW2. After 1 h, 50 μL of protein A–agarose (50%) was added to each mixture on ice. After another hour, the agarose was washed three times with high-salt buffer (10 mM Tris, pH 8.0; 500 mM NaCl; 0.1% NP-40; 0.05% BSA; 0.05% chicken egg albumin) and then boiled in 2× SDS sample buffer (Nicola *et al.,* 1998). Following SDS–PAGE, Western blots were probed with rabbit antisera R7 for HSV-1 gDt, R170 for PrV gDt, or EnvA for BHV-1 gDt.

Mass spectroscopy

Matrix-assisted laser desorption ionization mass spectrometry was performed in the linear mode on a Micromass TofSpec 2E time-of-flight mass spectrometer (1.0 m flight tube; Micromass, Beverly, MA) outfitted with an N2 (337 nm, 4 ns pulse) laser. Samples were diluted in matrices, using α-cyano-4-hydroxycinnamic acid as the matrix.

Glycosidase digestions

Purified PrV gDt, BHV-1 gDt, or HSV-1 gDt (1 μg) was denatured and treated with either 0.8 mU endoglycosidase H (Boehringer Mannheim, Indianapolis, IN) or 32 mU endoglycosidase F (Boehringer Mannheim) for 5 h at 37°C. The digestion products were analyzed by Western blot using an anti-tetra-His MAb.

Enzyme-linked immunosorbent assay (ELISA)

ELISA experiments were performed essentially as described previously (Whitbeck *et al.,* 1997). Microtiter plates were coated for 2 h with 50 μL of purified soluble receptor in PBS at a concentration of 4 μg/mL for HveAt or 10 μg/mL for HveBt or HveCt. Plates were washed with 0.1% Tween 20 in PBS and nonspecific binding was blocked by incubation with blocking solution. Serial dilutions of gDt (or other proteins) prepared in blocking solution were added for 2 h. The plates were washed and either MAbs (diluted 1:500 in blocking solution) or rabbit sera (diluted 1:1000) were added for 1 h to detect bound gDt. The plates were washed and HRP-conjugated goat anti-mouse or anti-rabbit antibodies were added for 30 min, washed again, and then rinsed with 20 mM citrate buffer (pH 4.5). ABTS peroxidase substrate (Moss, Inc., Pasadena, MD) was added and the absorbance at 405 nm was detected with a microtiter plate reader. Signals from identically treated wells that lacked receptor were subtracted from the results to account for background.

Optical biosensor analysis

Surface plasmon resonance experiments (Karlsson *et al.,* 1991; Myszka, 1999) were carried out essentially as described previously (Krummenacher *et al.,* 1999; Rux *et al.,* 1998; Whitbeck *et al.,* 1999; Willis *et al.,* 1998b) on a BIAcore X optical biosensor (BIAcore AB, Uppsala, Sweden) at 25°C. The running buffer was HBS-EP (BIAcore) containing 0.005% Tween 20. Flow cell 2 (Fc2) of a CM5 sensor chip was activated, HveCt was coupled via primary amines, and the flow cell surface was blocked. Between 1510 and 1750 resonance units (RU) of HveCt were immobilized for each chip used. Flow cell 1 (Fc1)

was activated and blocked without the addition of protein. To characterize the binding of proteins to HveCt, the flow path included both flow cells, the flow rate was 50 μL/min, and the data collection rate was 5 Hz. Proteins were serially diluted in HBS-EP. Binding was allowed for 2 min and the wash was delayed 2 min to allow for a smooth dissociation curve. The chip surface was regenerated with short pulses of Na_2CO_3 (pH 9.5–10.5) until the response signal on both flow cells returned to baseline. Sensorgrams were corrected for nonspecific binding by subtracting the signal achieved on Fc1 from that on Fc2. The signal obtained when buffer alone was injected was then subtracted from the total signal. The sensorgrams were analyzed using BIAEvaluation software, version 3.0. Model curve fitting was performed using global fitting with a 1:1 Langmuir binding model with drifting baseline. This is the simplest model for the interaction between a receptor and ligand. It follows the equation $A + B \leftrightarrows AB$. The rate of association (k_{on}) is measured from the forward reaction and the rate of dissociation (k_{off}) measured from the reverse reaction: $k_{off}/k_{on} = K_D$. We discounted the effects of mass transport by running samples at different flow rates and ensuring signals were not flow-rate dependent. For BHV-1 gDt, a maximum k_{off} was estimated using the equation $\ln(R_0/R_n) = k_{off}(t_n - t_0)$, where R_0 is the response at time zero (t_0) of dissociation and R_n is the response at time n (t_n). The reported maximum k_{off} is equal to the slope of a linear fit to the initial second of dissociation of 16 μM BHV gDt.

Virus entry inhibition assay

PK15, M3A, or CHO-IEβ8 cells (as negative control) were plated in 96-well plates and grown to confluence overnight. Cells were chilled to 4°C and the medium was replaced with DMEM–5% FCS containing various concentrations of PrV gDt, HSV-1 gDt, or BSA. After rocking for 90 min at 4°C, HSV-1 hrR3 (m.o.i. = 0.5) or PrV BeBlu (m.o.i. = 5) was added, incubated for 90 min at 4°C, and then shifted to 37°C. After 5 h, the cells were washed with PBS and lysed in DMEM containing 0.5% NP-40. β-Galactosidase activity was measured by addition of cell lysate to substrate (CPRG; Boehringer Mannheim). Absorbance at 570 nm at multiple time points was detected using a microtiter plate reader, the slopes were recorded, and the results were plotted as percentages of controls in which no protein was added.

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